METHOD FOR OVERPRODUCING A SPECIFIC RECOMBINANT PROTEIN WITH *P. CINNABARINUS* MONOKARYOTIC STRAINS

The present invention relates to the use of monokaryotic strains of filamentous fungi of the species *Pycnoporus* of the basidiomycete group, for the implementation of a method for preparing a specific recombinant protein, said method being carried out by overexpression of the gene encoding for this protein in the abovementioned monokaryotic strain of *Pycnoporus*.

At present, two fungal models are preferentially used by the large industrial groups within the framework of the production of enzymes involved in plant biotransformations, such as the metalloenzymes. These are *Aspergillus*, and *Trichoderma*, which belong to the deuteromycete group. However, production yields using these models, in particular in the production of laccases, do not exceed 150 mg/l.

The present invention results from the demonstration by the Inventors of the fact that the transformation of monokaryotic strains of *P. cinnabarinus* deficient in laccase activity using vectors containing the gene encoding for this laccase and the expression of which is under the control of a promoter identical to the endogenous *pLac* promoter of the laccase of *P. cinnabarinus*, leads to an equivalent production of laccase as during the implementation of a method for overproducing laccase by induction of the endogenous promoter of this laccase by the action of ethanol on monokaryotic strains of *P. cinnabarinus* not deficient in laccase activity, and which equals one g/l.

Similar results have been obtained by the Inventors by using the *gpd* promoter, and the *sc3* promoter of *Schizophyllum commune,* instead of the abovementioned *pLac* promoter.

A subject of the present invention is a method for preparing a specific recombinant protein, said method being carried out by overexpression of the gene encoding for this specific protein in a monokaryotic strain of filamentous fungi of the species *Pycnoporus* of the basidiomycete group, and comprises:

- a stage of culturing the abovementioned monokaryotic strain of *Pycnoporus*, said strain being transformed using an expression vector containing the gene encoding for the specific recombinant protein, the expression of which is placed under the control of a promoter corresponding to an endogenous promoter of the abovementioned fungi,

or of a different promoter (also designated exogenous promoter), said promoter being constitutive or inducible,

- if appropriate a stage of induction of the abovementioned promoter, when the latter is inducible,

- the recovery, and, if appropriate, the purification of the specific recombinant protein, produced in the culture medium.

A more particular subject of the invention is a method as described above, characterized in that the monokaryotic strain of *Pycnoporus* used for the overexpression of the gene encoding for the specific recombinant protein, is as obtained by culturing the original dikaryotic strain at 30°C in the dark for 15 days, followed by a stage of exposure to daylight for 2 to 3 weeks at ambient temperature until the formation of fruiting organs corresponding to differentiated hyphas called basidia, within which karyogamy (fusion of nuclei) then takes place, followed by meiosis which leads to the formation of four sexual spores, or genetically different haploid basidiospores, which, after germination, produces a monokaryotic mycelium.

Advantageously, the monokaryotic strain of *Pycnoporus* used in the abovementioned method of the invention, is a strain of *Pycnoporus cinnabarinus*.

The specific recombinant proteins overexpressed within the framework of the implementation of the method according to the invention, correspond either to endogenous proteins of *Pycnoporus*, or to different exogenous proteins of the endogenous proteins of the strain of *Pycnoporus* used for the production of said proteins. In particular these exogenous proteins correspond to endogenous proteins of basidiomycetes other than *Pycnoporus*, such as the basidiomycete enzymes involved in plant biotransformations, or correspond to endogenous proteins of strains of *Pycnoporus* different from the strain of *Pycnoporus* used for the production of said proteins.

A more particular subject of the invention is a method as described above, characterized in that the specific recombinant proteins correspond:

- to the following endogenous proteins of *Pycnoporus*:

  * the metalloenzymes, such as laccase, or tyrosinase,

  * or cellobiose dehydrogenase, xylanase, β-glycosidase, invertase, or α-amylase,

- to the exogenous proteins chosen from the following:

  * the tyrosinases of strains of *Pycnoporus* different from the strain of *Pycnoporus* used for the production of said proteins, such as the tyrosinase of

Pycnoporus sanguineus when the strain of *Pycnoporus* used for the production of this tyrosinase is different from *Pycnoporus sanguineus*,

* the laccases of basidiomycetes other than *Pycnoporus*, such as the laccase of *halocyphina villosa* (halophilic basidiomycete),

* the cinnamoyl esterases A (number EMBL Y09330) and B (number EMBL ANI309807) of *Aspergillus niger*.

Advantageously, in particular in the case of the preparation of specific recombinant proteins corresponding to the endogenous proteins of *Pycnoporus*, the monokaryotic strain of *Pycnoporus* used is deficient in the gene encoding for the endogenous protein to which the specific recombinant protein corresponds, in order not to have to separate the specific recombinant protein from the endogenous protein to which it corresponds during the purification of said recombinant protein.

As a variant, in particular in the case of the preparation of specific recombinant proteins corresponding to the endogenous proteins of *Pycnoporus*, the monokaryotic strain of *Pycnoporus* used may not be deficient in the gene encoding for the endogenous protein to which the specific recombinant protein corresponds, said strain then being transformed using an expression vector containing the gene encoding for the specific recombinant protein labelled in order to distinguish it from the endogenous protein during the purification stage. By way of illustration, the specific recombinant protein can be labelled by a histidine label (His-tag).

A more particular subject of the invention is therefore a method for preparing recombinant laccases corresponding to the endogenous laccases of *Pycnoporus*, characterized in that it comprises:

- a stage of culturing a monokaryotic strain of *Pycnoporus*, if appropriate deficient in the gene encoding for the endogenous laccase of *Pycnoporus*, transformed using an expression vector containing the gene encoding for a laccase of *Pycnoporus*, if appropriate labelled, and the expression of which is placed under the control of a promoter corresponding to the endogenous promoter of this laccase,

- a stage of induction of the abovementioned promoter, in particular by adding ethanol, or agricultural by-products containing lignocellulose such as wheat straw, corn bran and beet pulp, or compounds with an aromatic ring such as 2,5-xylidine, veratrylic acid, guaicol, veratrylic alcohol, syringaldazine, ferulic acid, caffeic acid and the lignosulphonates,

- the recovery, and, if appropriate, the purification of the recombinant laccase, if appropriate labelled, corresponding to the abovementioned endogenous laccase of *Pycnoporus* produced in the culture medium, in particular according to the method described in Sigoillot J.C., Herpoel I., Frasse P., Moukha S., Lesage-Meessen L., Asther M. 1999; Laccase production by a monokaryotic strain *Pycnoporus cinnabarinus* derived from a dikaryotic strain; World Journal of Microbiology and Biotechnology 15, 481-484.

The invention relates more particularly to a method as defined above, for preparing the recombinant laccase corresponding to the endogenous laccase of *Pycnoporus cinnabarinus* represented by SEQ ID NO: 2, characterized in that it comprises:

- a stage of culturing a monokaryotic strain of *Pycnoporus cinnabarinus*, if appropriate deficient in the gene encoding for the endogenous laccase of *Pycnoporus cinnabarinus*, transformed using an expression vector containing the nucleotide sequence (or nucleic acid) SEQ ID NO: 1 encoding for the recombinant laccase represented by SEQ ID NO: 2, if appropriate labelled, in particular by a His-tag label, and the expression of which is placed under the control of the *pLac* promoter corresponding to the endogenous promoter of the abovementioned laccase, the sequence of said *pLac* promoter being represented by SEQ ID NO: 3,

- a stage of induction by ethanol of the abovementioned *pLac* promoter,

- the recovery, and, if appropriate, the purification of the recombinant laccase, if appropriate labelled, represented by SEQ ID NO: 2 produced in the culture medium, in particular according to the method described in Sigoillot J.C., et al. (1999) mentioned above.

A more particular subject of the invention is a method for preparing recombinant laccases corresponding to the endogenous laccases of *Pycnoporus*, characterized in that it comprises:

- a stage of culturing a monokaryotic strain of *Pycnoporus,* if appropriate deficient in the gene encoding for the endogenous laccase of *Pycnoporus*, transformed using an expression vector containing the gene encoding for a laccase of *Pycnoporus* the expression of which is placed under the control of an exogenous promoter chosen from:

* the *gpd* promoter of the expression of the gene encoding for the glyceraldehyde 3-phosphate dehydrogenase of *Schizophyllum commune*, the nucleotide sequence of which is represented by SEQ ID NO: 4,

* or the *sc3* promoter of the expression of the gene encoding for the hydrophobin of *Schizophyllum commune*, the nucleotide sequence of which is represented by SEQ ID NO: 5,

- the recovery, and, if appropriate, the purification of the recombinant laccase corresponding to the abovementioned endogenous laccase of *Pycnoporus* produced in the culture medium, in particular according to the method described in Sigoillot J.C., et al. (1999) mentioned above.

The invention relates more particularly to a method as defined above, for preparing the laccase corresponding to the endogenous laccase of *Pycnoporus cinnabarinus* represented by SEQ ID NO: 2, characterized in that it comprises:

- a stage of culturing a monokaryotic strain of *Pycnoporus cinnabarinus*, if appropriate deficient in the gene encoding for the endogenous laccase of *Pycnoporus cinnabarinus*, transformed using an expression vector containing the nucleotide sequence SEQ ID NO: 1 encoding for the recombinant laccase represented by SEQ ID NO: 2, if appropriate labelled, in particular by a His-tag label, the expression of which is placed under the control of the exogenous gpd or sc3 promoter,

- the recovery, and, if appropriate, the purification of the recombinant laccase, if appropriate labelled, represented by SEQ ID NO: 2 produced in the culture medium, in particular according to the method described in Sigoillot J.C., et al. (1999) mentioned above.

A more particular subject of the invention is a method as defined above, for preparing recombinant tyrosinase corresponding to the tyrosinase of *Pycnoporus sanguineus* represented by SEQ ID NO: 16, characterized in that it comprises:

- a stage of culturing a monokaryotic strain of *Pycnoporus cinnabarinus* transformed using an expression vector containing the nucleotide sequence SEQ ID NO: 15 encoding for the recombinant tyrosinase represented by SEQ ID NO: 16, if appropriate labelled, the sequence SEQ ID NO: 15 being advantageously preceded by the nucleotide sequence delimited by the nucleotides situated at positions 128 and 190 of SEQ ID NO: 1 encoding for the peptide signal of *Pycnoporus cinnabarinus* delimited by the first 21 amino acids of SEQ ID NO: 2, and the expression of which is placed under the control of the *pLac* promoter corresponding to the endogenous promoter of the laccase of *Pycnoporus cinnabarinus*, the sequence of said *pLac* promoter being represented by SEQ ID NO: 3,

- a stage of induction by ethanol of the abovementioned *pLac* promoter,

- the recovery, and, if appropriate, the purification of the recombinant tyrosinase, if appropriate labelled, represented by SEQ ID NO: 16 produced in the culture medium.

The invention relates more particularly to a method as defined above, for preparing recombinant laccase corresponding to the laccase of *halocyphina villosa* represented in Figure 12 (SEQ ID NO: 18), characterized in that it comprises:

- a stage of culturing a monokaryotic strain of *Pycnoporus cinnabarinus*, if appropriate deficient in the gene encoding for the endogenous laccase of *Pycnoporus cinnabarinus*, transformed using an expression vector containing the nucleotide sequence represented in Figure 12 (SEQ ID NO: 17) encoding for the recombinant laccase represented by SEQ ID NO: 18, if appropriate labelled, and the expression of which is placed under the control of the *pLac* promoter corresponding to the endogenous promoter of the laccase of *Pycnoporus cinnabarinus*, the sequence of said *pLac* promoter being represented by SEQ ID NO: 3,

- a stage of induction by ethanol of the abovementioned *pLac* promoter,

- the recovery, and, if appropriate, the purification of the recombinant laccase, if appropriate labelled, represented by SEQ ID NO: 18 produced in the culture medium.

A subject of the invention is also the nucleotide sequence encoding for the *pLac* promoter of the endogenous laccase of *Pycnoporus cinnabarinus*, and corresponding to the sequence SEQ ID NO: 3, or any sequence derived from this promoter by substitution, addition or suppression of one or more nucleotides and retaining the property of being a promoter of the expression of sequences.

The invention also relates to any expression vector, such as the plasmid pELP, characterized in that it comprises the sequence SEQ ID NO: 3 of the abovementioned *pLac* promoter, or a derived sequence as defined above.

A more particular subject of the invention is any expression vector as defined above, characterized in that it comprises a gene encoding for a specific recombinant protein, and the expression of which is placed under the control of the abovementioned *pLac* promoter, or of a derived sequence as defined above.

The invention relates more particularly to any expression vector as defined above, characterized in that the specific recombinant protein is a protein corresponding:

- to the following endogenous proteins of *Pycnoporus*:

  * the metalloenzymes, such as laccase, or tyrosinase,

  * or cellobiose dehydrogenase, xylanase, β-glycosidase, invertase, or α-amylase,

- to the exogenous proteins chosen from the following:

* the tyrosinases of strains of *Pycnoporus* different from the strain of *Pycnoporus* used for the production of said proteins, such as the tyrosinase of *Pycnoporus sanguineus* when the strain of *Pycnoporus* used for the production of this tyrosinase is different from *Pycnoporus sanguineus*,

* the laccases of basidiomycetes other than *Pycnoporus*, such as the laccase of *halocyphina villosa* (halophilic basidiomycete),

* the cinnamoyl esterases A and B of *Aspergillus niger*.

The invention also relates to any host cell transformed using an expression vector as defined above.

A more particular subject of the invention is any abovementioned host cell, corresponding to monokaryotic cells of strains of *Pycnoporus,* such as strains of *Pycnoporus cinnabarinus*.

A subject of the invention is also the use of expression vectors as defined above, or of abovementioned host cells, for the implementation of a method for overproducing a specific recombinant protein as defined above.

The invention is further illustrated by means of the following detailed description of the PCES: *Pycnoporus cinnabarinus* Expression System, namely the development of an efficient model of fungal expression making it possible to get rid of the industrial models currently used by the large European groups (*Aspergillus* and *Trichoderma*).

In summary, this is a system of eukaryotic expression and more specifically of filamentous fungi of the basidiomycete group, *Pycnoporus cinnabarinus*, which has been developed by the Inventors for the overexpression of proteins of industrial interest. This work was carried out within the framework of the study of metalloenzymes, such as the laccases, and has in particular made it possible to clone the genes involved in their overexpression, and overproduction of the laccases in large quantities using fermenters, in order to use them in industrial applications for food use (bread making, preparation of drinks in order to modulate the colour of tea, assist in the clarification of fruit juices and alcoholic drinks, formation of agropolymers) and non-food use (treatment of "jeans", degradation of aromatic pollutants in soil, bio-bleaching of lignocellulose fibres in the field of papermaking pulp).

I) Obtaining monokaryotic lines of *Pycnoporus cinnabarinus* for the transformation of the fungus and the overproduction of genes of interest.

The purpose of this stage is to isolate then select the haploid cell lines originating from sexual spores of a filamentous fungus, *Pycnoporus cinnabarinus*, which are used at times as host for the expression of the genes of interest. *P. cinnabarinus* is a heterothallic fungus which is found in the wild state in the dikaryotic form (two non-paired nuclei per cell) from which monokaryotic lines are selected (one nucleus per cell), which are potentially more stable and can therefore be used for genetic transformation. Within the framework of this study the Inventors undertook to select monokaryotic lines deficient in laccase activity (lac^-). In the dikaryotic state, the fungus can multiply by vegetative route (Fig. 1). But, under the influence of particular environmental conditions, it is possible, in the laboratory, to induce the formation of fruiting organs. Within differentiated hyphas called basidia, karyogamy (fusion of the nuclei) then took place, followed by meiosis which leads to the formation of four sexual spores, or genetically different haploid basidiospores. After germination, each basidiospore produces a monokaryotic mycelium. A simple colorimetric test then makes it possible to select only strains devoid of laccase activity.

1) Isolation of the monokaryotic strains

The fruiting medium is composed of 2% malt extract (W/V) and agar (1.6% W/V). The cultures are seeded in Petri dishes and kept at 30°C in the dark for 15 days before being exposed to daylight for 2 to 3 weeks at ambient temperature. The fruiting body appears orange-red. The monospores are then harvested with sterile water on the lid of the Petri dish. The suspension is diluted and cultured in Petri dishes containing an MA2 medium (2% malt W/V and 2% agar W/V) for the purpose of isolating colonies. Isolated pure cultures are seeded and kept in MA2 medium at 30°C for 5 days and stored at 4°C.

Under these conditions, a monokaryotic strain deficient in laccase activity was selected for transformation with the expression vector for the purpose of overexpressing the laccase gene. A Southern blot study was carried out and made it possible to demonstrate that this strain is deficient in the gene encoding for laccase in *P. cinnabarinus*.

2) Rapid test for detecting laccase activity of monospore colonies

A piece of mycelium is placed in a Petri dish and covered with a drop of 0.1% syringaldazine (W/V) in ethanol solution; After 15 minutes, a change in colour is observed. The 2,2-azino-bis-[3-ethylthiazoline-6-sulphonate] (ABTS) can also be used as substrate in order to reveal laccase activity.

3) Cultures conditions for producing laccase

An inoculum is removed from the precultures which have been growing for 10 days at 30°C in Roux flasks containing 200 mL of a synthetic medium with the following composition for 1L : maltose (20 g), diammonium tartrate (1.84 g), disodium tartrate (2.3 g), KH_2PO_4 (1.33 g), $CaCl_2$, H_2O (0.1 g), $MgSO_4$, $7H_2O$ (0.5 g), $FeSO_4$,7H2O (0.07 g), $ZnSO_4$,$7H_2O$ (0.046 g), $MnSO_4$,H_2O (0.035 g), $CuSO_4$,$5H_2O$ (0.1 g), yeast extract (1 g), vitamin solution (1 mL/L) according to Tatum et al. (Biochemical mutant strains of *Neurospora* produced by physical and chemical treatment. American Journal of Botany, 37: 38-46, 1950). The mycelium from two flasks is collected, mixed with 100 mL of sterile water and homogenized with an Ultraturax mixer for 60 seconds. In order to produce laccase, the synthetic medium is inoculated with 1 mL of the mycelium suspension. The medium (100 mL) is then incubated at 30°C in baffled 250-mL Erlenmeyer flasks under stirring (120 rpm).

II) Cloning of the gene encoding for the laccase of *Pycnoporus cinnabarinus* and its promoter with a view to the construction of an expression vector

This involves a eukaryotic expression system and more particularly of filamentous fungus, *Pycnoporus cinnabarinus*, of the basidiomycete group for the overproduction of specific recombinant proteins. The study model selected is that of the laccase of *P. cinnabarinus*. At present, two fungal models are preferentially used by the large industrial groups. These are *Aspergillus* and *Trichoderma* which belong to the Deuteromycete group. This expression system is therefore completely original and should bridge the gap with regard to development of basidiomycete expression systems compatible with industrial requirements (possibility of large-scale production of proteins secreted in the extracellular medium and culture of the producer fungus in a fermenter).

1) Cloning of the Pycnoporus cinnabarinus laccase gene and its promoter

In a first stage, the Inventors amplified a fragment of the gene encoding for laccase using degenerated nucleotide primers (Fig. 2). The upstream F2 (SEQ ID NO: 6; CAYTGGCAYGGRTTCTTCC) and downstream R8 (SEQ ID NO: 7; GAGRTGGAAGTCRATGTGRC) degenerated primers were deduced, respectively, from the copper I and IV binding regions of the laccases of related organisms and used in a PCR reaction (Polymerase Chain Reaction) using the genomic DNA of *P. cinnabarinus* I-937. 100 ng of genomic DNA; 0.2 mM of dATP, dCTP, dTTP, and dGTP; 25 pmol of each nucleotide primer; 0.1 volume of 10X *Pfu* polymerase buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3) and 1 U of *Pfu* polymerase are added to 10 µl of reaction mixture. The mixture is heated at 94°C for 5 minutes before adding the polymerase. The reaction conditions are the following: 5 cycles at 94°C, 5 minutes; 55°C, 30 seconds; and 72°C, 4 minutes; then 25 cycles at 94°C, 30 seconds; 55°C, 30 seconds, and 72°C, 3 minutes. A stage of 10 minutes at 72°C is carried out in order to complete the reaction. A 1.64 kpb band was obtained corresponding to the central part of the laccase gene. The DNA sequence was cloned in pGEM-T in order to sequence this part of the gene.

By a Southern blot technique (Fig. 3), we defined the restriction sites suitable for obtaining a minimum DNA fragment, being able to contain the whole of the laccase gene, and which are capable of serving to amplify the missing 5' and 3' ends. A Southern blot was carried out with the genomic DNA of *P. cinnabarinus* with the *Bam*HI, *Eco*RI, *PstI*, *PvuII*, *Sac*I, *Sma*I and *Xba* I enzymes and made it possible to select *PstI* which produces a 3.5 kpb band by digestion of the genomic DNA. In order to amplify the missing parts of the gene, an inverse PCR technique was used with a mixture of PCR containing nucleotide primers specific to the central part previously isolated and the genomic DNA of *P. cinnabarinus*. The PCR reaction is carried out with 150 ng of DNA cleaved by *PstI* and looped back on itself by ligation and the nucleotide primers Fex (SEQ ID NO: 8; GGATAACTACTGGATCCGCG) and Rex (SEQ ID NO: 9; CGCAGTATTGCGTGGAGAG). The reaction conditions are the following: 5 cycles at 94°C, 5 minutes; 55°C, 30 seconds; and 72°C, 5 minutes; then 25 cycles at 94°C, 30 seconds; 55°C, 30 seconds, and 72°C, 4 minutes with a final stage of 10 minutes at 72°C. The amplified DNA fragment corresponds to a 2.7 kpb band which was cloned in pGEM-T and sequenced.

The whole of the gene encoding for laccase was then defined by combining the central part and the amplified 5' and 3' parts. In order to verify this sequence, the whole of the gene was amplified (3.331 kpb, Fig. 4) with the nucleotide primers Fin (SEQ ID NO: 10; GACATCTGGAGCGCCTGTC) and Rin (SEQ ID NO: 11; ATCGAAGGTTCCGATGACTGACATGAC) from the genomic DNA of *P. cinnabarinus*. This gene was also cloned from the genomic DNA of *P. cinnabarinus* ss3 and proved to be identical to that isolated from *P. cinnabarinus* I-937.

2) Construction of the expression vector using the laccase gene promoter

Starting with the laccase gene sequence, the Inventors cloned the promoter of this gene using the same strategy used previously to isolate the gene, i.e. with an inverse PCR technique on a fragment of genomic DNA (3.5 kpb) cleaved this time by the restriction enzyme *Bgl*II (Fig. 5). Two thousand five hundred and twenty seven kpb in front of the laccase gene were thus cloned by inverse PCR and sequenced. This promoter was placed in a vector with a resistance to ampicillin for its sub-cloning in the bacterium and a resistance to the phleomycin used as a selection marker in the fungus. A terminator of the gene encoding for the hydrophobin sc3 of *Schizophyllum commune* was placed downstream in order to terminate the transcription stage. This vector called pELP is used for the homologous expression of laccase (Fig. 6). Two other heterologous promoters were used in this study. These are the promoters of the genes encoding for the glyceraldehyde 3-phosphate dehydrogenase (gpd) and hydrophobin (sc3) of *Schizophyllum commune* (Fig. 6), constituting the expression vectors pEGT and pESC respectively. The whole of the nucleotide sequences of vectors pEGT (SEQ ID NO: 12), pESC (SEQ ID NO: 13), and pELP (SEQ ID NO: 14), are to be found in Figures 7, 8 and 9 with the positions of promoter, selection-marker and terminator.

III) Transformation of the monokaryotic strain with the expression vectors (study model: the laccase of *Pycnoporus cinnabarinus*)

1) Preparation of the mycelium for obtaining protoplasts

A quarter of a colony cultured in solid medium (10 days) is homogenized with a mixer (Ultraturax type, slow speed) for one minute in 50 ml of YM medium (per litre: glucose 10 g, peptone 5 g, yeast extract 3 g, malt extract 3 g). The homogenate is transferred to a sterile 250-ml Erlenmeyer, to which 50 ml of YM medium is added,

then incubated at 30°C and under stirring (225 rpm) for 20 hours. The culture is once again homogenized for 1 minute (slow speed) and 100 ml of YM medium is added. The homogenate is transferred to a 500-ml Erlenmeyer and cultured overnight at 30°C.

2) Preparation of the protoplasts

The fungus culture is centrifuged for 10 minutes at 2000 rpm in an oscillating rotor (50 ml tube). 16 g (moist weight) are washed in 40 ml of a 0.5 M $MgSO_4$ or 0.5 M saccharose solution. In the case where saccharose is used, the lytic enzyme used in order to digest the walls is diluted in the saccharose. The mycelium is then centrifuged for 10 minutes at 2000 rpm and the supernatant eliminated. As regards the lysis of the fungal walls, 10 ml of lytic enzyme (Glucanex, Sigma) diluted 1 mg/ml in a 0.5 M $MgSO_4$ solution is added to the mycelium originating from 50 ml of culture. Digestion takes place in a 500-ml Erlenmeyer at 30°C under gentle stirring over 3 to 4 hours. During this incubation, the appearance of the protoplasts is monitored with a microscope. Ten ml of sterile water are added, then mixed gently. The protoplasts are left for 10 minutes, the time taken for water equilibrium to occur (the protoplasts will float on the surface). They are then centrifuged for 10 minutes at 2000 rpm in an oscillating rotor. The supernatant containing the protoplasts is gently transferred into a new 50 ml of solution. The remaining pellet can be re-incubated with 25 ml of a 0.5M $MgSO_4$ solution in order to recover the maximum amount of protoplasts (the centrifugation stage is then repeated). A volume of 1 M sorbitol, equal to that of the protoplast preparation, is added to it. For 10 minutes, the protoplasts are left to release water. This preparation is then centrifuged for 10 minutes at 2000 rpm. The supernatant is eliminated, leaving a little sorbitol. The protoplasts are transferred into a new tube. The previous tube is rinsed with the 1M sorbitol solution and the protoplasts recovered, added to the new tube. The protoplasts are counted and centrifuged for 10 minutes at 2000 rpm. They are then diluted to a concentration of 2. 10^7 protoplasts per ml in the 1M sorbitol solution. A 0.5 M CaCl2 solution (1/10) is added to the protoplasts.

3) Transformation of the protoplasts

For the transformation, 100 µl of protoplasts are transformed with 5 to 10 µg of vector (maximum volume of 10 µl) in a sterile 10 ml tube. They are then incubated for 10 to 15 minutes in ice. A volume of a 40% PEG 4000 solution is added, then mixed and the protoplasts are incubated for 5 minutes at ambient temperature. Two and a half

ml of regeneration medium (for 100 ml: glucose 2 g, $MgSO_4$, $7H_2O$ 12.5 g, KH_2PO_4 0.046 g, K_2HPO_4 0.1 g, bacto peptone 0.2 g, yeast extract 0.2 g) are added to the protoplasts which are incubated overnight at 30°C. Selection dishes (YM medium containing 7 µg/ml phleomycin, square dishes) are preheated at 37°C. Seven and a half ml of a top agar mixture (1% Low Melting Point agarose diluted in a YM medium containing 7 to 10 µg/ml phleomycin) are added to the regeneration medium containing the protoplasts and are poured into the preheated selection dishes. When the top agar solution has solidified, the dishes are incubated at 30°C for 4 days. The transformants are then transferred to new selection dishes.

4) Targeting the transformants

Starting with 16 g of mycelium, approximately 1 to 2.10^7 protoplasts are generally obtained. The regeneration percentage is 10 %. As regards the vector pESC, the monokaryons were transformed with the vector containing the cDNA (BRFM 472, 473 and 474) or the gene encoding for the laccase of *P. cinnabarinus* (BRFM 470 and 471) (Fig. 10). In parallel, other monokaryons were transformed with the promoters pEGT (GPD11, 12 and 13) or with the vector pELP (12.3, 12.7 and 12.8) containing the gene encoding for the laccase (Fig. 10). In view of the results two transformants emerge from the batch with equivalent activities, the transformants 12.7 and GPD14. The activity over time was monitored for the transformants GPD14 and 12.7 (Fig. 11). The activity is detectable from 3-4 days and increases up to 12 days to reach approximately 1200 nkatal/ml i.e. 72000 U/l with the addition of ethanol to the culture medium.

Legends to the figures

Figure 1: Isolation of monokaryotic strain deficient in laccase activity.

Figure 2: Isolation of the gene encoding for the laccase of *Pycnoporus cinnabarinus* laccase.

Figure 3: Southern blot study of the gene encoding for the laccase of *Pycnoporus cinnabarinus.*

Figure 4: Sequence of the gene encoding for the laccase of *Pycnoporus cinnabarinus*.

Figure 5: Sequence of the pLac promoter sequence of the gene encoding for the laccase of *Pycnoporus cinnabarinus* (up to the ATG encoding for the methionine of the laccase).

Figure 6: Restriction map of the three expression vectors pEGT, pESC, pELP, used for the production of laccase in *Pycnoporus cinnabarinus*.

Figure 7: Nucleotide sequence of the vector pEGT, containing the gpd gene promoter (4480-5112), a phleomycin resistance marker (507-1822) and the sc3 gene terminator (71-507).

Figure 8: Nucleotide sequence of the vector pESC, containing the sc3 gene promoter (1-1033), a phleomycin resistance marker (1540-2855) and the sc3 gene terminator (1104-1540).

Figure 9: Nucleotide sequence of the vector pELP, containing the laccase gene (promoter 4457-6983), a phleomycin resistance marker (507-1822) and the sc3 gene terminator (71-507)

Figure 10: Results of production of the transformants having the most significant activities. The culture was carried out with or without (control) ethanol.

Figure 11: Monitoring of the laccase activities of the transformants GPD 14 and 12.7 as a function of time with or (control) without ethanol.

Figure 12: Sequence of the gene encoding for the laccase of *halocyphina villosa*.